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Regeneration of oral siphon pigment organs in the ascidian *Ciona intestinalis*

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ABSTRACT

Ascidians have powerful capacities for regeneration but the underlying mechanisms are poorly understood. Here we examine oral siphon regeneration in the solitary ascidian *Ciona intestinalis*. Following amputation, the oral siphon rapidly reforms oral pigment organs (OPO) at its distal margin prior to slower regeneration of proximal siphon parts. The early stages of oral siphon reformation include cell proliferation and re-growth of the siphon nerves, although the neural complex (adult brain and associated organs) is not required for regeneration. Young animals reform OPO more rapidly after amputation than old animals indicating that regeneration is age dependent. UV irradiation, microcautery, and cultured siphon explant experiments indicate that OPOs are replaced as independent units based on local differentiation of progenitor cells within the siphon, rather than by cell migration from a distant source in the body. The typical pattern of eight OPOs and siphon lobes is restored with fidelity after distal amputation of the oral siphon, but as many as 16 OPOs and lobes can be reformed following proximal amputation near the siphon base. Thus, the pattern of OPO regeneration is determined by cues positioned along the proximal distal axis of the oral siphon. A model is presented in which columns of siphon tissue along the proximal–distal axis below pre-existing OPO are responsible for reproducing the normal OPO pattern during regeneration. This study reveals previously unknown principles of oral siphon and OPO regeneration that will be important for developing *Ciona* as a regeneration model in urochordates, which may be the closest living relatives of vertebrates.

Introduction

Animals in many different phyla have the capacity to replace lost parts by regeneration (Morgan, 1901; Berrill, 1966; Brockes and Kumar, 2008; Bely and Nyberg, in press). Pluripotent stem cells, called neoblasts in planarians (Reddien and Sánchez Alvarado, 2004; Salo, 2006), play key roles in tissue and organ renewal and replacement (Hall and Watt, 1989). In chordates, the most powerful capacities for regeneration are found in the urochordate (tunicate) ascidians (Berrill, 1951). The regeneration of lost external parts, such as limbs and tails, is restricted to only a few vertebrate groups, most prominently the urodele amphibians (Nye et al., 2003). Humans and other mammals have relatively poor capacities for limb regeneration. Metaphylogenetic analysis has inferred urochordates as the closest living relatives of vertebrates (Bourlat et al., 2006; Delsuc et al., 2006; Vienne and Pontarotti, 2006). Therefore, studying ascidian regeneration is likely to improve our understanding of why tissue and organ regeneration has become more restricted during vertebrate evolution.

Ascidians are well known for mosaic embryonic development in which isolated parts of embryos lack the regulative potential to form a complete tadpole larva (Satoh, 1994). This feature is reversed after metamorphosis when juvenile and adult body parts attain the capacity to be replaced. Colonial ascidians, such as *Botryllus*, *Botrylloides*, *Perophora*, and *Clavellina*, which reproduce both sexually and asexually, are able to regenerate from small fragments or pluripotent blood cells (Brien, 1930; Berrill and Cohen, 1936; Freeman, 1964; Berrill, 1966; Rinkevich et al., 2007; Kawamura et al., 2008). In *Botryllus schlosseri*, stem cells originating in the anterior part of the endostyle proliferate and migrate over long distances to form new tissues and organs during regeneration and budding (Voskoboynik et al., 2008). Solitary ascidians, such as *Styela* and *Ciona*, which only reproduce sexually, show more limited abilities for regeneration, which is restricted to structures such as the siphons and neural complex (including the cerebral ganglion of the central nervous system) (Schultze, 1899; Hirschler, 1914; George, 1937; Sutton, 1953; Dahlberg et al., 2009). According to Hirschler (1914), these structures can regenerate in *Ciona intestinalis* only when internal organs, such as the heart, stomach, gonads, and at least a part of the pharynx (branchial sac) are present, suggesting that the latter could be sources of a diffusible factor(s) and/or stem/progenitor cells required for regeneration. In contrast to *Botryllus* (Voskoboynik et al.,

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2008), stem cells responsible for regeneration have not been identified in *Ciona*.

The siphons of *C. intestinalis* contain brightly colored orange pigment organs localized at their distal margins (Millar, 1953). These pigment organs are sometimes referred to as ocelli or photoreceptor organs, although their ability to detect light and transmit sensory information through neural connections has not been established (Hecht, 1918; Millar, 1953; Dilly and Wolken, 1973). Here we investigate the regeneration of ocellus-like pigment organs (oral pigment organs or OPO) in the oral siphon of *C. intestinalis*. The availability of an extensive molecular tool kit (Satoh et al., 2003) and transposon-mediated transgenic animals with GFP expression in the nervous system (Sasakura, 2007; Sasakura et al., 2007) makes *Ciona* an attractive model system to determine the principles of ascidian regeneration. The removal of one or both *Ciona* siphons leads to complete regeneration within about 1–2 months (Hirschler, 1914; Fox, 1924, Sutton, 1953; Whittaker, 1975). Most previous studies were concerned with the nutritional conditions and growth parameters leading to siphon regeneration, leaving the underlying mechanisms unknown. Basic unresolved questions include whether siphon regeneration recapitulates development, the source(s) of progenitor cells, if the pre-existing pattern is precisely reformed, and the potential role of the nervous system in this process. In this investigation, we address these and other longstanding issues in *Ciona* oral siphon and OPO regeneration as a first step in developing a mechanistic understanding of the powerful regenerative capacities of solitary ascidians.

Materials and methods

Biological materials

C. intestinalis was collected and maintained in running seawater at the Marine Biological Laboratory, Woods Hole, MA, USA or purchased from Station Biologique, Roscoff, France and maintained in running seawater at Station Biologique or in a closed culture system at Gif-sur-Yvette, France (Joly et al., 2007). The E[MiTSAdTPOG]15 (E15) enhancer trap transgenic line, which exhibits GFP fluorescence in the adult nervous system (Awazu et al., 2007), was maintained in the closed culture system at Gif-sur-Yvette, France.

Oral siphon amputation

Animals were anesthetized in 0.2–1 mg/ml tricaine methanesulphonate (MS222; Sigma Chemicals, St. Louis, MO) in seawater for about 15 min at 18 °C. The tunic was dissected from some animals prior to oral siphon amputation but since it was unnecessary for siphon or OPO regeneration, this step was not carried out in subsequent amputations. The oral siphon was amputated through planes perpendicular or oblique to its proximal–distal axis by severing with fine dissection scissors or straight-bladed sharp spring scissors (5 or 8 mm; Fine Science Tools, Inc, Foster City, CA). More than 90% of the animals recovered after a single cycle of oral siphon amputation. With further cycles, the ability to survive decreased to 50–60% by the fourth cycle.

Analysis of OPO and siphon regeneration

Beginning about 1 h after amputation, regenerating animals were observed every 12–18 h, and at bi-weekly intervals after 10 days post-amputation (dpa) for a total of 8 weeks. The extent of siphon regeneration was determined by visual inspection, photography, and counting the number of regenerated OPOs in anesthetized animals observed under a dissection microscope. In some cases, oral siphons were excised, fixed overnight in 4% paraformaldehyde (PFA), dehydrated through an ethanol series, cleared in xylene, embedded in Paraplast, and sectioned at 10 μm. The sections were attached to glass slides and examined by microscopy.

Fine-scale analysis of regeneration was carried out in flat mount preparations. To prepare flat mounts, all or part of the oral siphon was removed by making a cut in a plane perpendicular to the proximal–distal axis. The excised siphon was fixed for 1 h or overnight in 4% PFA; the tunic was removed; and the siphon cylinder was washed three times in PBS, and severed along its proximal–distal axis. The splayed siphon was mounted under a glass cover slip for observation and photography using a dissection or compound microscope. Flat mounts of the E15 enhancer trap line were viewed and photographed under a fluorescence microscope at 470 nm to detect GFP fluorescence.

Phalloidin and DAPI staining

To detect the receptor cell component of normal and regenerated OPOs, siphon flat mounts were double stained with phalloidin and DAPI. Splayed siphons, prepared as described above, were washed three times in PBS (10 min), incubated in PBS containing 0.1% Triton X-100 (30 min), washed three times in PBS (10 min), and incubated at room temperature with 25 μg/ml rhodamine-phalloidin (Molecular Probes, Eugene, OR) and 0.01 μg/ml DAPI (Molecular Probes) in the dark for 1 h. After staining, the specimens were washed three times in PBS (10 min), and flat mounts were prepared and viewed using a fluorescence dissecting or compound microscope.

EdU and phospho-histone H3 labeling of mitotic cells

Cell proliferation was detected by incorporation of 5-ethynyl-2′-deoxyuridine (EdU) into DNA and by staining mitotic cells with phospho-histone-3 antibody. Regenerating animals were incubated with 200 μM EdU (Click-iT™ EdU Alexa Fluor® 488 or 594 High-Throughput Imaging HCS Assay, Invitrogen, Carlsbad, CA) in 100 ml seawater for 16 h immediately after oral siphon amputation and chased in seawater without EdU for the remainder of the experiment. Unoperated controls were incubated with EdU for 16 h. Whole mounts of EdU treated animals were fixed with 4% PFA, washed three times in PBS containing 0.1% Tween-20 (PBST), then stained with a 1:50 dilution of phospho-histone H3 antibody (Upstate Biotechnology Inc., Lake Placid, NY) for 1.5 h, and the primary antibody was detected with a 1:400 dilution of goat anti-rabbit secondary antibody coupled to Alexa 594 (Molecular Probes) for 1.5 h. The specimens were rinsed 3 times in PBST and post-fixed in 4% PFA for 20 min at room temperature. Alexa Fluor azide 488 was used to detect EdU positive cells according to Dahlberg et al (2009). When used on transgenic animals (see Fig. 5N), the detection of EdU positive cells was performed with Alexa Fluor Azide 594. The preparations were rinsed five times in PBT and flat mounts were prepared for photography.

Ultraviolet irradiation

Ultraviolet (UV) irradiation was carried out using a short-wave Mineral light Lamp (Model UVG-11, UVP Inc., San Gabriel, CA), which emitted maximally at 254 nm. The UV irradiation conditions are illustrated in Fig. 6. Animals ranging from 5 to 10 cm in length were immersed in plastic Petri dishes containing MS222 in seawater. The bilaterally symmetric animals were positioned with their left sides (oral siphon ventrally) facing the UV source, which was positioned 7.3 cm above the base of the Petri dish. A black plastic filter was inserted between the animal and the UV source, leaving the oral siphon exposed to UV irradiation and covering the remainder of the animal. The UV dose was determined empirically. UV irradiation was carried out for 5 min on the left side of the animal. Some animals were irradiated unilaterally, as described above, whereas others were irradiated globally. After an initial 5 min cycle of irradiation on the left side, the globally irradiated animals were flipped to their opposite sides and the right side of the oral siphon was subjected to a 5 min cycle of UV irradiation. Controls were irradiated for one cycle of 5 min

on the left side, flipped to the right side, and UV irradiated for another 5 min period while completely protected by the black plastic filter. After irradiation, animals were immersed in seawater lacking MS222 for recovery, and recovered animals were transferred to running seawater to monitor regeneration.

Microcautery

Instruments for microcautery were stout glass needles with a small glass sphere on the tip, which were pulled from Pasteur pipettes and shaped under the flame of a micro Bunsen burner. Anesthetized animals were subjected to microcautery under a dissection microscope. The instrument was heated to red color under the Bunsen burner flame, applied to the center of a single OPO, and moved laterally until the entire cluster of red pigment cells and associated epidermal regions disappeared from view. Microcauterized animals were moved to seawater lacking MS222 for recovery, and after 30 min recovered animals were placed in running seawater to monitor regeneration.

Oral siphon explant culture

Oral siphon explants were prepared as illustrated in Fig. 7A. An initial amputation near the distal siphon margin was immediately followed by a second amputation in a more proximal region of the siphon. These operations resulted in two cylindrical siphon explants: (1) a siphon tip explant with the original OPO, siphon lobes, and yellow pigment band on the distal side and the severed edge on its proximal side, and (2) a siphon mid-piece explant with severed edges on its distal and proximal sides. The siphon explants from single animals were co-cultured at 18 °C in 12 well plastic plates containing Millipore filtered seawater supplemented with 10% GIBCO Neurobasal culture medium (Invitrogen, Carlsbad, CA), 10 μg/ml kanamycin, and 50 μg/ml gentamycin. The culture medium was replaced daily. Under these conditions, most siphon explants survived for about 10 days. OPO regeneration was monitored periodically during the culture period.

Neural complex removal

A small hole was made in the tunic between the oral and atrial siphons and the neural complex was excised with sharp forceps (Dahlberg et al., 2009). The operated animals were checked to be certain of complete removal of the neural complex, which is visible as a glistening white structure in living animals. Sham-operated animals were dissected as described above but the neural complex was not removed. Following siphon amputation, animals were placed in seawater lacking MS222 for 30 min to recover, and then the recovered animals were returned to seawater for regeneration. The operated animals were periodically checked for neural complex replacement during the OPO regeneration period.

Results

Structure and development of siphon pigment organs

The distal margins of the oral and atrial siphons contain ocellus-like pigment organs located in notches between the siphon lobes (Figs. 1A–E). Each pigment organ consists of a crypt-like epithelium of columnar receptor cells and an underlying cup-shaped aggregation of mesenchymal orange pigment cells (Figs. 1C–E). In transgenic animals of the E15 enhancer trap line, which express GFP in the nervous system (Awazu et al., 2007), GFP staining was detected in the basal portion of the receptor cells (Figs. 1F, G), suggesting that this region could function as a neural component (also see Dilly and Wolken, 1973). Bands of yellow (*Ciona* from Woods Hole and Roscoff) or orange (E15 *Ciona* strain from Japan) pigment cells lie between the pigment organs along the distal margins of the siphons (Figs. 1A–C, F). A zone of circular muscle fibers lies immediately below the ring of pigment and siphon lobes. Longitudinal muscle bands (LMBs) are present along the proximal distal axis of the oral and atrial siphon, diffusing in a zone of circular muscle at their distal margins, and tapering at their bases. Areas mostly free of muscle fibers, called ORBs (oral pigment organ regeneration bands) in the oral siphon, are positioned adjacent to the LMBs (see Figs. 2S, T). The most proximal portion of the oral siphon contains a large transverse blood vessel and a ring of tentacles, which project into the oral cavity (see Fig. 8A). The oral siphon ends at the dorsal edge of the branchial sac, which includes the tip (or hood) of the endostyle (Fig. 8A). Both siphons are richly innervated by nerve fibers originating from the cerebral ganglion, a part of the neural complex located between the two siphons (see Fig. 8F).

The number of pigment organs was counted in the oral and atrial siphons of 51 animals ranging in length from 2–16 cm. The atrial siphon of each animal had six pigment organs. In contrast, the oral siphon of most animals had eight pigment organs (43 animals or 84%), although some animals had seven (7 animals or 14%) or nine pigment organs (1 animal or 2%) (also see Millar, 1953). The number of oral and atrial siphon lobes corresponds to the number of pigment organs.

Chiba et al. (2004) followed pigment cell formation in the developing siphons up to the so-called second ascidian stage. In the last few stages that were studied, the pigment organ precursors appeared as clusters of un-pigmented cells, which eventually turned orange, as siphon development advanced. This study was extended here by following later stages of pigment organ development in the oral siphon of juveniles (Figs. 1H–J). In young juveniles, eight small foci of orange pigment cells were spaced equidistantly below notches in the developing oral siphon lobes (Fig. 1H). As in adults, seven or nine rather than the typical eight pigment foci were observed in a few animals. In intermediate sized juveniles, the foci of orange pigment cells merged with the notches to form pigment organs between the siphon lobes, and yellow pigment cells eventually appeared as wing–like projections along the siphon margins (Fig. 1I). Eventually, the zone of yellow pigment cells extended bilaterally from each pigment organ and connected around the siphon circumference to form the yellow pigment band characteristic of adults (Fig. 1J).

OPO regeneration

In this study, we focused on the regeneration of pigment organs (oral pigment organs or OPO) in the oral siphon. The oral siphon was amputated above the major traverse blood vessel and row of tentacles (Fig. 2A), and regeneration was assessed periodically in living and fixed animals. The results shown in Figs. 2 and 3 were obtained for OPO regeneration in average-sized (4–7 cm) adults. As described previously (Whittaker, 1975), regeneration of the oral siphon to full length took about 1.5 to 2 months (Figs. 2B–F). However, the OPO and siphon lobes regenerated much more rapidly, usually within 8–10 days after amputation (Figs. 2G–N).

The following sequence of events was observed during OPO regeneration. By 1-day post amputation (dpa), the wound surface was repaired, as indicated its smooth edge. At this time, the regenerating siphon did not contain any obvious concentrations of orange or yellow pigment cells, although single pigment cells were scattered more proximally in the remaining part of the siphon, most prominently in the ORB (Figs. 2H, O, P). Beginning at about 2–3 dpa, lines of orange pigment cells, interspersed between regions lacking them, were observed along the margin of the regenerating oral siphon (Figs. 2I, P, Q, S). The lines of pigment cells were located immediately above the ORB and did not extend into adjacent regions above the LMB (Fig. 2S). By 3–4 dpa, the orange pigment lines condensed into spots

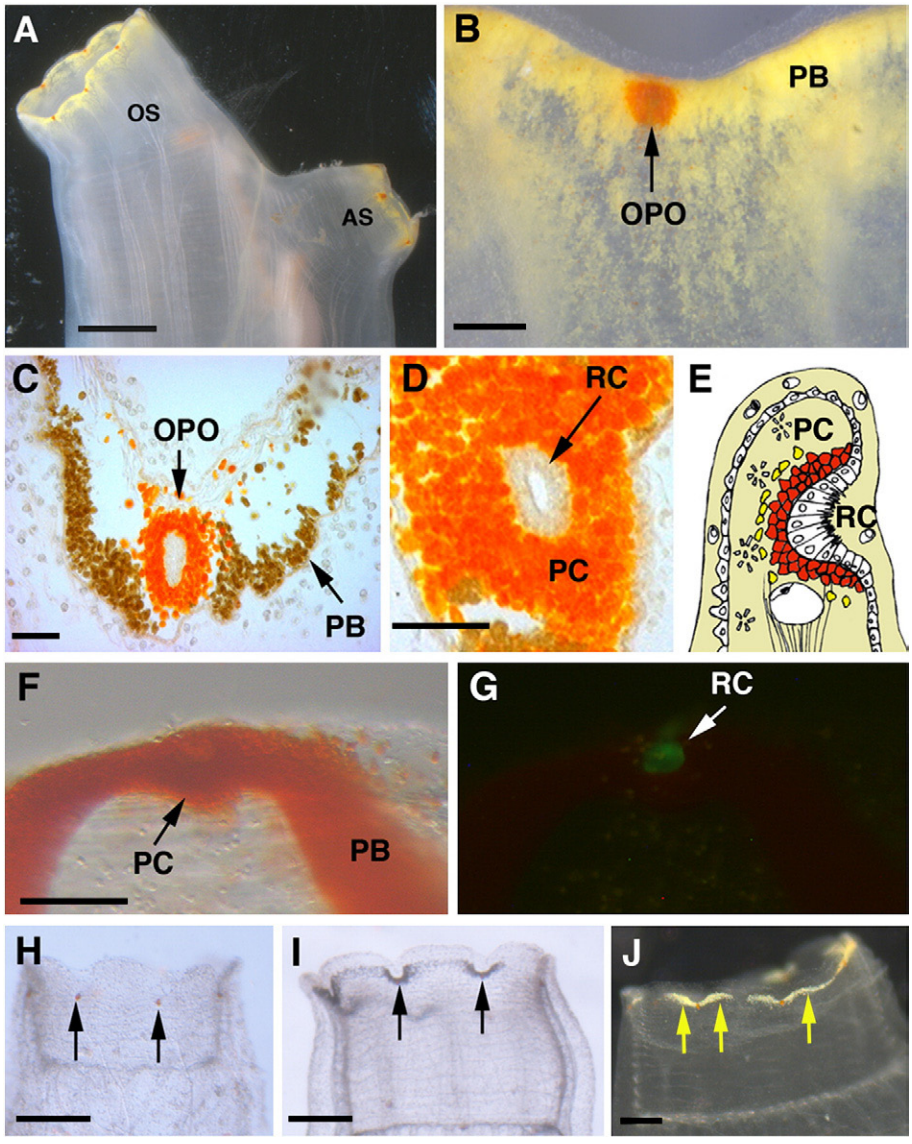

Fig. 1. Oral siphon structure and development. (A–G) Adults. (A) An adult without the tunic showing OPOs, siphon lobes, and yellow pigment bands around the circumference of the oral siphon (OS) and atrial siphon (AS). Scale bar: 4 mm. (B) A single OPO located in a notch between two oral siphon lobes surrounded by yellow pigment bands (PB). Scale bar: 100 μm. (C, D) Sections through an OPO showing the orange pigment cell cup (PC), receptor cells (RC), and yellow pigment bands (PB) located in a notch between the oral siphon lobes. Scale bars in panels C and D are 50 μm. (E) Diagram of an OPO in cross-section showing the relationship between the orange pigment cup (PC) and receptor cells (RC). The OPO faces the outside of the siphon (right). Modified from von Haffner (1933). (F, G) DIC (F) and fluorescence (G) images of OPO in an E15 enhancer trap line animal showing GFP-labeled receptor cells (RC). The PB is orange, rather than yellow, in E15 animals. Scale bar in panel F is 100 μm; magnification is the same in panels F and G. (H–J) Juveniles. (H) A small jjuvenile with orange pigment spots (arrows). (I) A larger juvenile showing orange pigment spots (arrows) surrounded by "wings" of yellow pigment cells beginning to form the pigment band within the notches between siphon lobes. (J) A late juvenile with a zone of yellow pigment cells (arrows) extending from the OPOs along the siphon margin to join the yellow pigment cells extending from an adjacent OPO. Scale bars in panels H–J are 150 μm.

(Figs. 2J, R, T), which were positioned above the center of each ORB (Fig. 2T). By about 7 dpa, the orange pigment spots became organized as pigment cups (Fig. 2K; also see Figs. 3K, N). About 10–15 dpa masses of yellow pigment cells (orange in the E15 transgenic line) first appeared as wing-like projections from each new OPO (Fig. 2L). Subsequently, the number of yellow pigment cells increased, forming wing-shaped masses, which eventually connected to re-produce the yellow pigment band along the oral siphon margin (Figs. 2M, N). Thus, regeneration of the OPO and marginal pigment band appears to recapitulate the steps of their original development (see Figs. 1H–J).

Fig. 3 shows the regeneration sequence of different parts of the OPO determined using several different markers. Orange pigment cup regeneration was determined by DIC microscopy, receptor cell regeneration was followed by staining with phalloidin (a marker of basally distributed actin filaments) (Figs. 3B, E), and DAPI (a marker of polarized basal nuclei) (Fig. 3C) (Dilly and Wolken, 1973), and regeneration of the putative neural component of the OPO was determined by GFP fluorescence in E15 transgenic animals (Figs. 3D, E). Orange pigment spots were reformed by about 3 dpa (Figs. 3F–J). By 5–6 dpa, the orange pigment spots became organized into cups (Figs. 3F, K, P, I, N, S). At this time receptor, cells were also detectable in epidermal crypts, but they were not strongly labeled by phalloidin (Figs. 3G, L), DAPI (Figs. 3H, M), or GFP (Figs. 3J, O). Strong rhodamine, DAPI, and GFP labeling appeared simultaneously at about 8–12 dpa (Figs. 3Q, R, T). Thus, we conclude that orange pigment cup formation precedes terminal receptor cell differentiation based on delayed expression of phalloidin, DAPI, and GFP markers.

Up to this point, the new oral siphon extended only a short distance distal to the initial point of amputation (Figs. 2C, D). Once the OPOs and marginal yellow pigment were reformed, however,

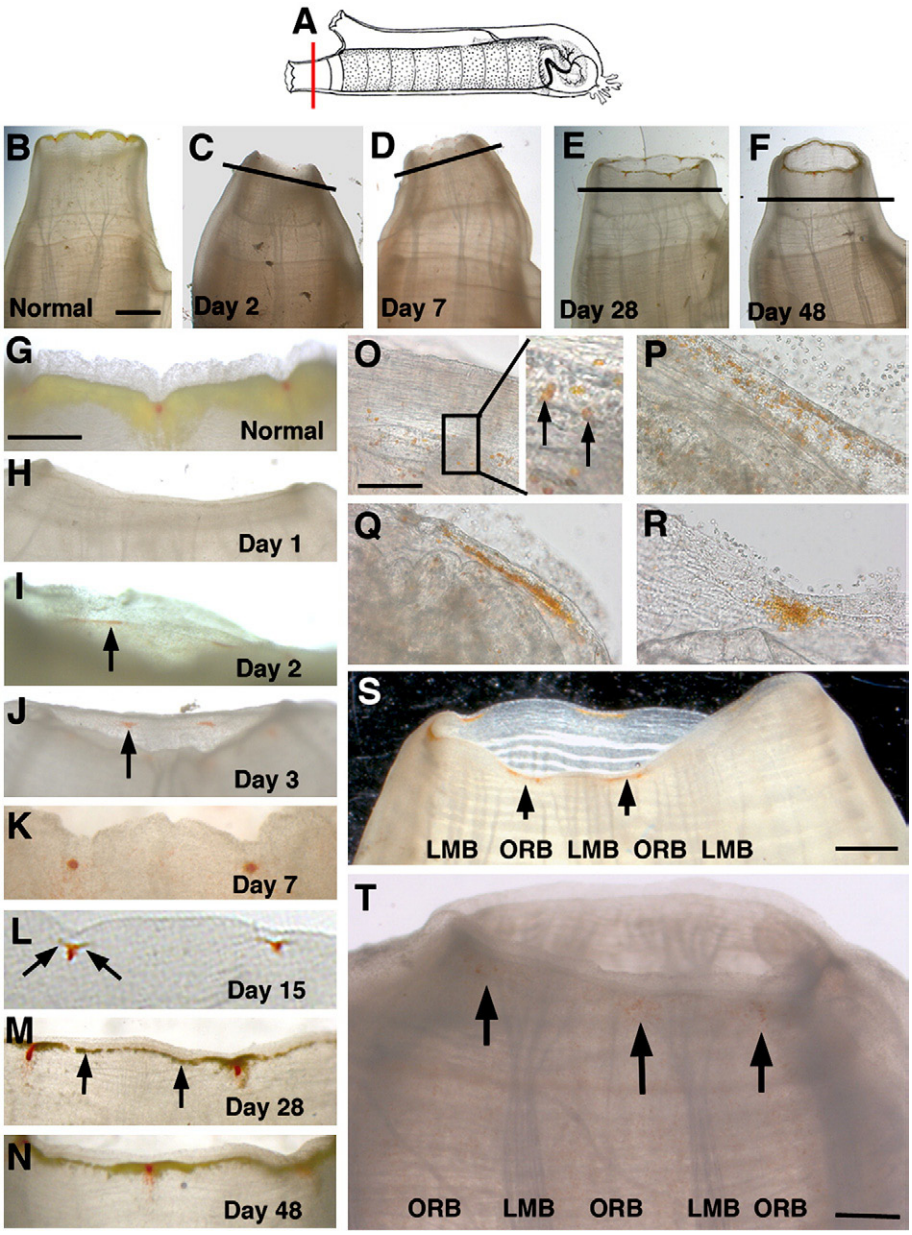

Fig. 2. Oral siphon and OPO regeneration. (A) A diagram showing the approximate position of oral siphon amputation. (B) A normal oral siphon, with OPO, siphon lobes, and yellow pigment bands. (C–F) Oral siphon regeneration. Black lines below the siphon margins indicate the approximate position of the amputation planes. Different animals are shown at each day. Scale bar in panel B is 4 mm; magnification in the same in panels B–F. (G–N) OPO, siphon lobe, and yellow pigment band regeneration. (G) A normal siphon prior to amputation. (H) At 1 day after amputation, the distal margin of the siphon is smooth but no OPO or siphon lobes have regenerated. (I) At 2 days after amputation, lines (arrow) of orange pigment cells appear at intervals along the margin of the regenerating siphon. (J, K) Between 3 and 7 days after amputation, the lines of orange pigment cells condense into spots (arrow, J), and new siphon lobes begin to appear between them (K). (L) At 15 days after amputation, "wings" of yellow pigment cells (slanting arrows) are formed adjacent to each new OPO. (M) At 28 days after amputation, the yellow pigment cells are more numerous and are in the process of connecting between OPOs (arrows). (N) At 48 days after amputation, OPO, yellow pigment band, and oral siphon regeneration are complete. Scale bar in panel G is 500 μm; magnification is the same in panels G–N. (O–R) Orange pigment spot regeneration in flat mount preparations. (O) At 1 day after amputation, orange pigment cells are scattered in the siphon wall (see arrows in 4× inset on the right). (P) At 2 days after amputation, orange pigment cells begin to accumulate at the distal margin of the regenerating siphon. (Q) At 3 days after amputation, lines of orange pigment cells are present along the siphon margin. (R) At 4 days after amputation, orange pigment spots are concentrated below notches between the re-forming siphon lobes. Subsequent stages of OPO regeneration are shown in Figs. 3F–T. Scale bar in panel O is 50 μm; magnification is the same in panels O–R. (S, T) Orange pigment spot regeneration is related to pre-existing oral siphon structure. (S) At about 2 days after amputation, lines of orange pigment cells (arrows) form above longitudinal muscle-free areas (the OPO regeneration band or ORB, see text) located adjacent to longitudinal muscle bands (LMB). (T) At about 3 days after amputation, orange pigment cells (arrows) begin to aggregate above the approximate midpoint of every ORB. Scale bars in panels S and T are 200 μm.

proximal growth gradually increased the length of the oral siphon to its original size (Figs. 2E, F). Regeneration of the oral siphon and OPO proceeded at about the same rate when the tunic was present or removed, indicating that this structure is unnecessary for oral siphon or OPO regeneration (data not shown). We conclude that the distal parts of the oral siphon are regenerated prior to reforming its proximal parts and that OPO development and regeneration are strikingly similar.

OPO regeneration decreases with age

Because of differences noted in the timing of OPO regeneration in animals of different size, we asked whether there is a relationship between the rate of OPO regeneration and age. In *Ciona*, age is correlated with overall length of the animal (Berrill, 1947; Millar, 1952). Therefore, to determine the relationship between age and OPO regeneration, we amputated the oral siphons of 66 animals ranging in

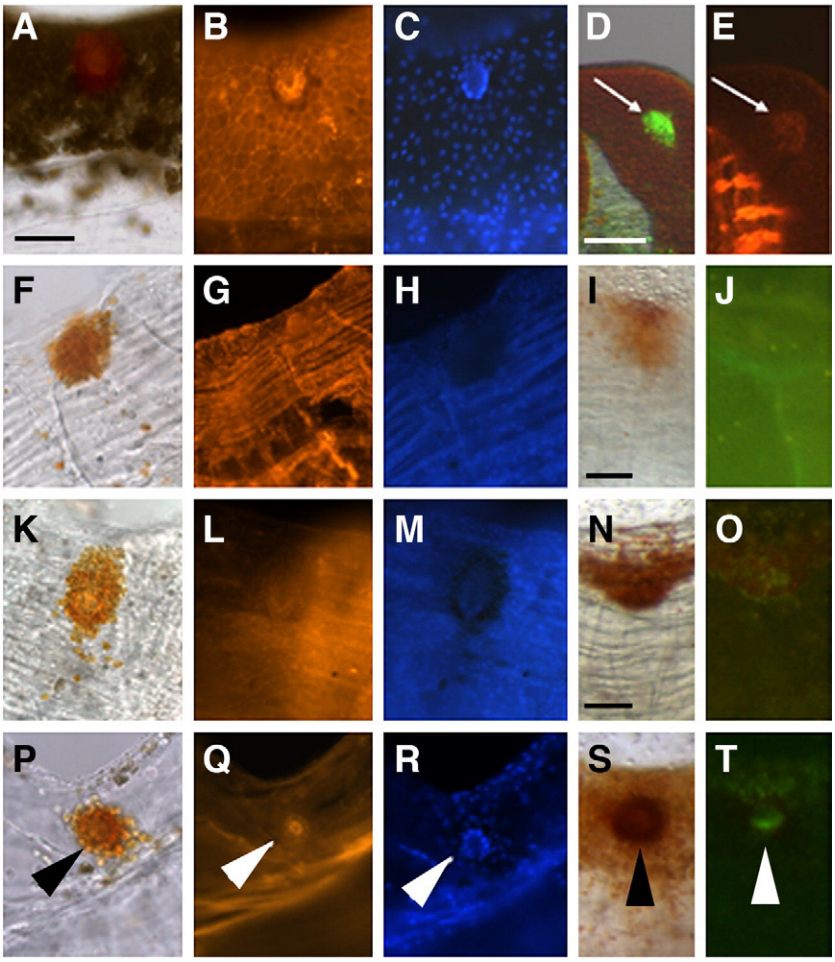

Fig. 3. Flat mount preparations showing regeneration of the orange pigment cup and receptor cells. OPO structure prior to amputation (A–E) and 3 (F–J), 5–6 (K–O), and 8–12 (P–T) days after amputation. (A, F, K, P) DIC images. (B, G, L. Q) Rhodamine-phalloidin staining showing receptor cell regeneration in the pigment cup (arrowhead in Q). (C, H, M, R) DAPI staining showing the regeneration of receptor cells in the pigment cup (arrowhead in R). Panels A–C, F–H, K–M, and P–R are the same animals. Scale bar in panel A is 100 μm; magnification is the same in panels A–R. (D, E) GFP-labeled (D) and rhodamine-phalloidin stained (E) receptor cells in E15 transgenic animals. Scale bar in panel D is 100 μm; magnification is the same in panels D and E. (I, J, N, O, S, T) Regeneration of GFP stained receptor cells in E15 transgenic animals. DIC images (I, N, S) and fluorescence (J, O, T) in the same animals with arrowheads showing the ventral margin of the pigment cups. Scale bars in panels I and N are 100 μm; magnification is the same in panels N–T.

length from 2 to 15 cm (Fig. 4), the regenerating animals were cultured individually, and the time (dpa) required for orange pigment cups to re-appear was determined. As shown in Fig. 4, a negative correlation was revealed between animal size and the rate of OPO regeneration: smaller (younger) animals regenerated OPO more rapidly than larger (older) animals. Dahlberg et al. (2009) also reported that neural complex regeneration proceeds more slowly in larger *Ciona*, suggesting a decline in overall regeneration capacity related to age.

Cell proliferation during siphon regeneration

Cell proliferation during oral siphon regeneration was investigated by EdU incorporation into DNA and labeling of mitotic cells with phospho-histone H3 antibody. In unoperated controls, EdU incorporation was detected in the oral siphon wall (Fig. 5A), the ring of oral siphon tentacles (Fig. 5B), the branchial sac and endostyle (Fig. 5C), and the neural complex/dorsal tubercle (Fig. 5D). To determine whether cell proliferation occurs during OPO regeneration, the oral siphon was amputated (Fig. 2A), regenerating animals were immediately incubated in EdU for 16 h, EdU was chased with seawater, and the animals were fixed and EdU incorporation was determined at 2, 4, 6, and 7 dpa (Fig. 5). Quantification of EdU-labeled cells in 200 μm^2 regions of normal and regenerating siphons is shown in Fig. 5O. The EdU-treated animals were then stained for phospho-histone H3

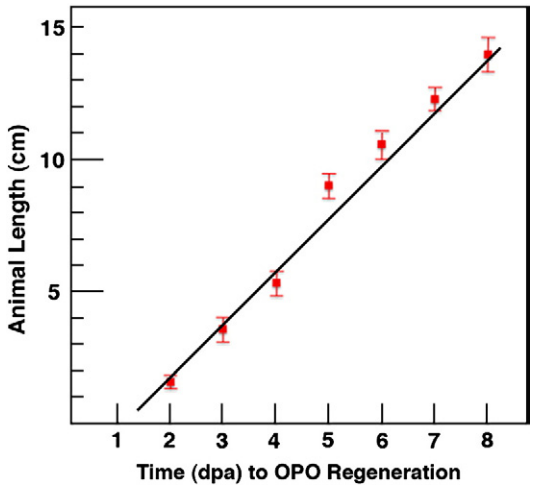

Fig. 4. OPO regeneration time increases with age. The oral siphons of animals ranging in body length from 2–15 cm were amputated (Fig. 2A) and the length (age) of animals with reformed orange pigment spots was determined daily for up to 8 days post amputation (dpa). The red points represent mean animal length (age) with reformed orange pigment spots at 2–8 dpa. The bars represent standard error of the mean for animals with reformed orange pigment spots at each dpa. N = 10 at 2 dpa, 11 at 3 dpa, 8 at 4 dpa, 9 at 5 dpa, 5 at 6 dpa, 14 at 7 dpa, and 9 at 8 dpa. The sloping line indicates the line of best fit through the points.

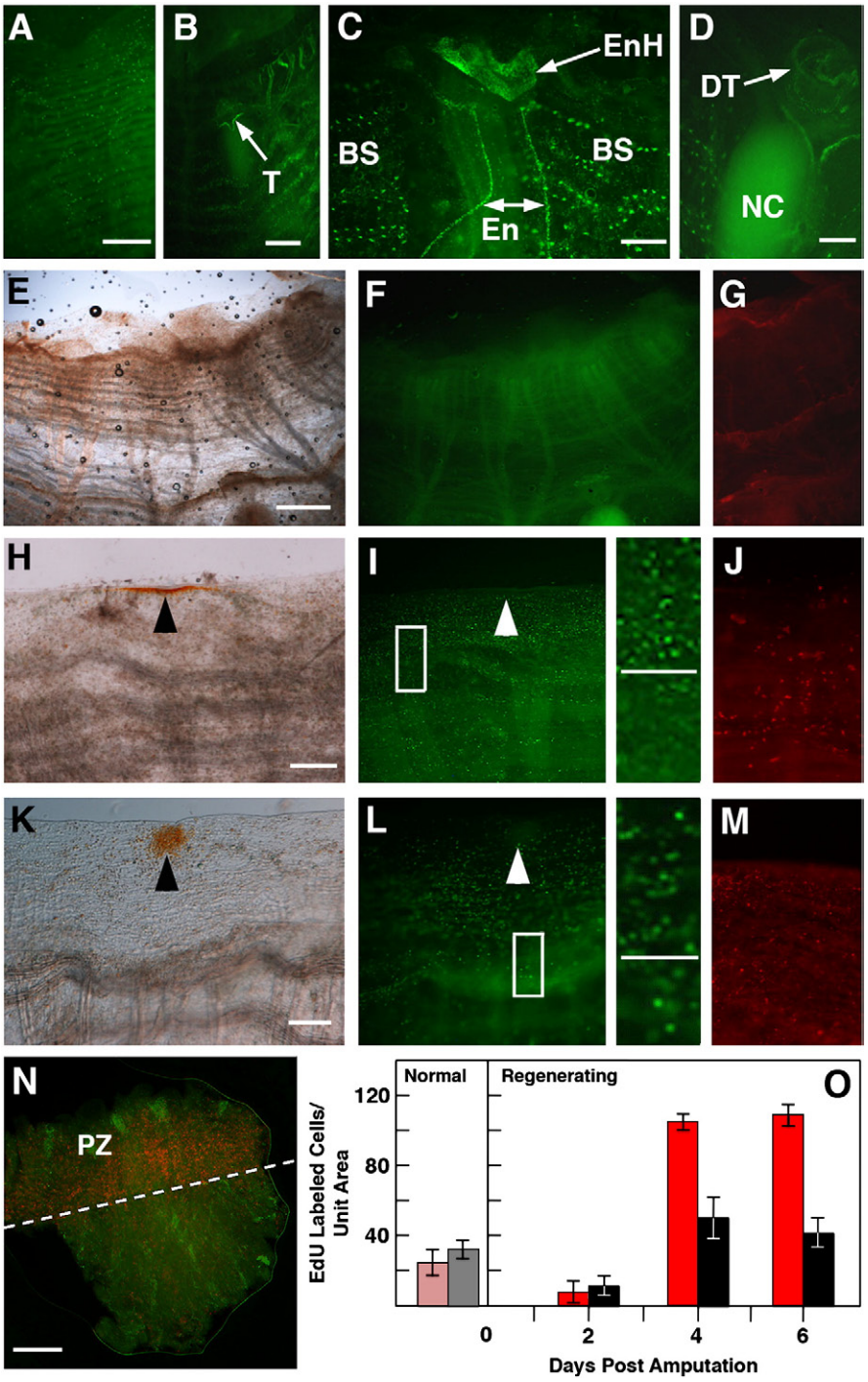

Fig. 5. Cell proliferation during oral siphon regeneration. (A–D) EdU-labeled cells in the oral siphon (A), oral siphon tentacles (B), endostyle and branchial sac (C), and the neural complex and dorsal tubercle (D) in un-amputated controls after a 16 h labeling period. T, Tentacles; BS, branchial Sac; En, endostyle; EnH, hood of the endostyle; DT, dorsal tubercle; NC, neural complex. Scale bars: 200 μm in panel A, 500 μm in panel B, 200 μm in panels C and D. (E, F, H, I, K, L). Bright field (E, H, K) and corresponding fluorescence (F, I, L) images of EdU-labeled cells in the regenerating oral siphon at 2 (E, F), 4 (H, I), and 6 (K, L) days after amputation. EdU was administered for 16 h beginning immediately after amputation. Arrowheads: Orange pigment line (H, I) and spot (K, L) in bright field photographs. Panels I and L have insets (white boxes) at 4× magnification shown immediately to the right. The white lines in the insets represent the approximate site of the original amputation planes. (G, J, M) Fluorescence images of phospho-histone H3-labeled cells in the regenerating oral siphon at 2 (G), 4 (J), and 6 (M) dpa. Scale bar in panel E is 50 μm; magnification is the same in panels E–M. (N) EdU labeling in the oral siphon of a E15 transgenic animal at 7 dpa showing concentration of EdU-labeled cells (red) above of the original plane of amputation, which is shown by a dashed line. PZ, proliferation zone. Scale bar: 100 μm. Panels A–N are flat mount preparations. (O) Histogram showing quantification of EdU-labeled cells in the regenerating oral siphon at 2, 4, and 6 dpa in the right hand frame. EdU-labeled cells were counted in 200 μm^2 areas. In the left hand frame of the histogram, the mean EdU-labeled cell number per until area is shown in distal (pink bar) and proximal (gray bar) regions of control unoperated oral siphons. In the right hand frame of the histogram, the mean EdU-labeled cell number per unit area is shown in the regions above (red bars) and below (black) the original amputation planes in regenerating animals. Error bars represent standard errors of the mean. Statistical analysis using Student's *t* test showed that the differences in EdU labeling above and below the original amputation site are significant ($P<0.01$, $N=3$) at 4 and 6 dpa.

expression. At 2 dpa, the level of EdU-labeled cells was reduced in both the growing distal and residual proximal parts of regenerating siphons, relative to normal siphons and siphons at later stages of regeneration (Figs. 5A, E–F, O). Phospho-histone H3 labeling was also low at 2 dpa (Fig. 5G), suggesting that amputation may temporarily depress cell division throughout the siphon. By 4 dpa and 6 dpa,

however, the number of EdU-labeled cells was much higher in the distal relative to the proximal region of the siphon (Figs. 5E, F, H, I, K, L, O). An increase in phospho-histone H3-labeled cells was also seen at 4 and 6 dpa relative to 2pa (Figs. 5G, J, M). Fig. 5N shows that by 7 dpa EdU-labeled cells were concentrated in a proliferation zone above the original amputation plane in the regenerating siphon. We did not observe EdU or phospho-histone H3 labeling of orange pigment cells in regenerating siphons (Figs. 5H–M), implying that there may be an undetectably low number of cell divisions prior to their differentiation or that they may differentiate without multiplying. The EdU and phospho-histone H3 experiments indicate that cell proliferation occurs during OPO and oral siphon regeneration.

Effects of UV irradiation and microcautery on OPO regeneration

To investigate the effect of UV irradiation on OPO regeneration, the oral siphon was irradiated globally or unilaterally prior to amputation using the shielding designs shown in Fig. 6. UV irradiation of fully shielded controls had no effect on OPO regeneration (Figs. 6A, B). When assessed at 8–10 dpa, global irradiation blocked OPO regeneration around the entire circumference of the siphon (Figs. 6C, D), whereas unilateral UV irradiation inhibited OPO regeneration exclusively on the irradiated side of the siphon (Figs. 6E, F). Next, a rectangular area of tissue located on one side of the oral siphon about 0.5–1 cm below its distal margin was UV irradiated prior to amputation of the distal end

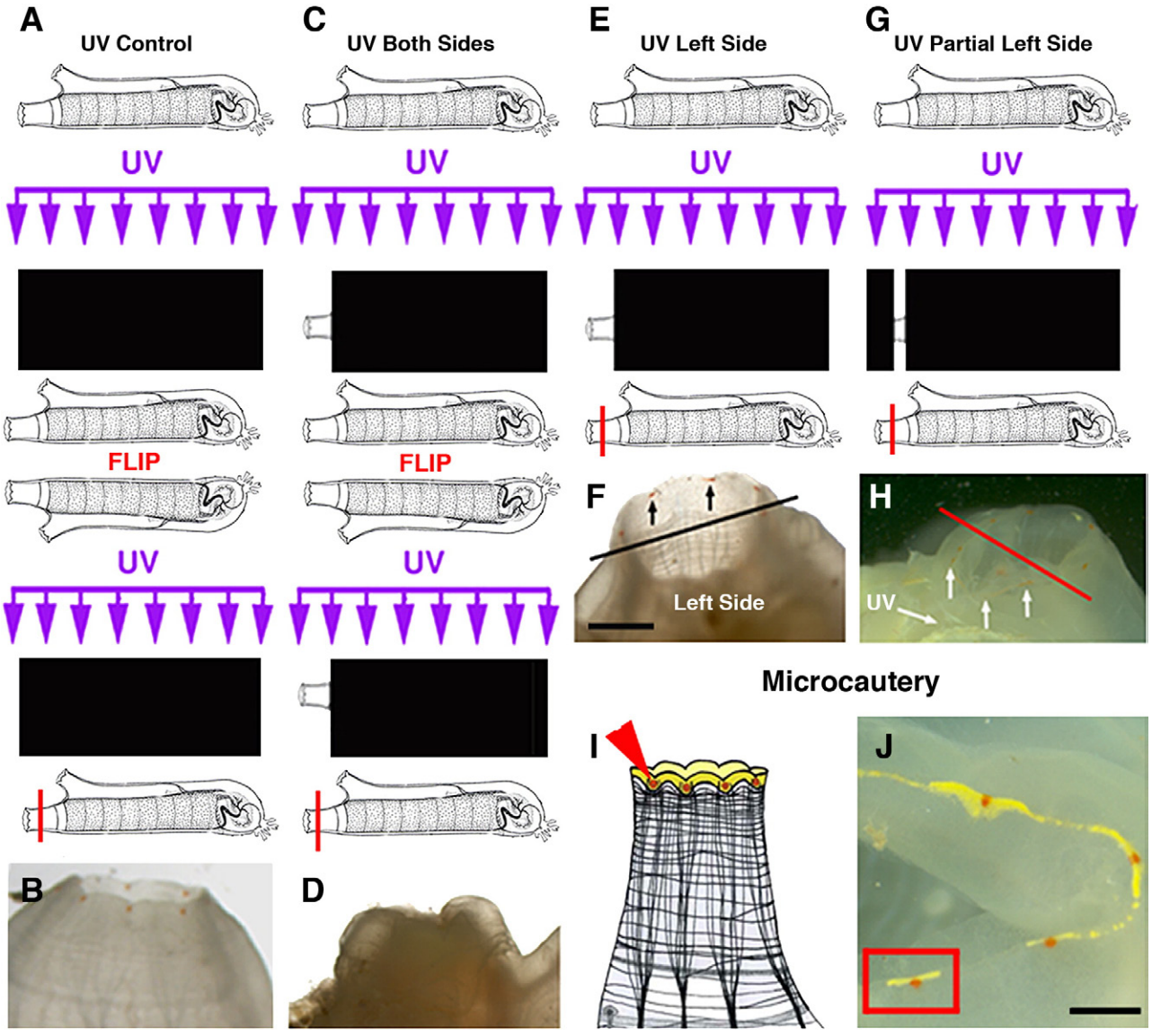

Fig. 6. Effects of UV irradiation and microcautery on OPO regeneration. (A–H) UV irradiation. (A, C, E, G) Diagrams illustrating the UV irradiation and shielding procedures. (A and below) Control for global UV irradiation in which animals were UV irradiated while completely shielded on the left side, then flipped to the right side and UV irradiated again prior to amputation. (C and below) Global UV irradiation was carried out by the same two-part UV irradiation procedure prior to amputation as in panel A but shielding did not cover the distal half of the oral siphon. (E and below) Unilateral UV irradiation on the left side of the oral siphon carried out prior to amputation by shielding as shown. (G and below) Unilateral UV irradiation on the left side was restricted to a region of the oral siphon about 1–2 cm below the distal margin carried out by shielding as shown prior to amputation. (B, D, F, H) Examples of results obtained at 6 days post-amputation in animals subjected to the UV irradiation procedures in panels A, C, E, and G, respectively. (B, D) OPO regeneration was blocked after global UV irradiation (D), but controls regenerated OPOs (B). (F) OPO regeneration was blocked on the left side of the oral siphon (arrows) after unilateral UV irradiation on the left side. The diagonal black line in panel F indicates the approximate border between the left (radiated and un-regenerated) and right (non-irradiated and regenerated) sides of the oral siphon. (H) OPO regeneration occurred (arrows) after UV irradiation of a restricted area below the amputation site. A portion of the UV irradiated area is show by the arrow labeled UV. The diagonal red line in panel H represents the approximate border between the regenerating OPO with or without a UV irradiated area below them. Scale bar in panel F is 200 μm; magnification is the same in panels B, D, F, and H. (I–J) Microcautery. (I) A diagram of the oral siphon indicating the position of microcautery. (J) A new OPO, including an orange pigment cup and yellow pigment "wings," appeared in horizontal register along the margin of the oral siphon (red box) in approximately the same position as the original OPO within 12 days after microcautery. Scale bar in panel J: 200 μm.

(Fig. 6G), leaving a small strip of intact living tissue between the UV irradiated area and the wounded margin. The results show that OPO regeneration occurred normally under these conditions, including the marginal zone immediately above the UV irradiated area (Fig. 6H). Thus, the strip of living siphon tissue between the amputated margin and the lesion created by UV irradiation is sufficient to support OPO regeneration. The unilateral UV irradiation results also suggest that OPOs may be able to regenerate independently of each other. To further explore this possibility, regeneration was assessed after single OPOs were destroyed by microcautery (Fig. 6I). In every case, new OPOs and adjacent yellow pigment bands regenerated in the same position as the original OPOs (Fig. 6J). The results show that OPOs are capable of regenerating as independent units.

OPO regeneration in oral siphon explants

The results of the UV irradiation experiments can be interpreted in two ways. First, progenitor cells could reside in the oral siphon below pre-existing OPO. Second, the oral siphon region located below each OPO may be required for regeneration, perhaps as a precursor cell niche, but progenitor cells could migrate into this region from outside the oral siphon. Possible sources of OPO progenitor cells outside the siphon could be the endostyle (Voskoboynik et al., 2008), the branchial sac (Hirschler, 1914), or other tissues that are active in cell proliferation (Figs. 5A–D).

To distinguish between these possibilities, OPO regeneration was investigated in oral siphon explants (Fig. 7). To produce these explants, the oral siphon was first amputated immediately below the preexisting OPO and then a second amputation was done in the siphon below the first amputation site (Fig. 7A). These operations created two cylindrical siphon explants, a tip explant containing the siphon rim with OPOs and a mid-section explant without OPO, which were co-cultured for up to 10 days. The following results were obtained in 17 of 32 (53%) cultured explants; the other explants either did not show OPO regeneration or perished in culture. In mid-section explants, a large number of orange pigment cells appeared in vertical stripes within the ORB during the first few days in culture (Figs. 7F, G, J), and by about 5–6 days pigment spots were observed on the distal but not the proximal margin of these explants (Figs. 7B–I, K bottom). The number of pigment spots that appeared in the siphon mid-section explants corresponded to the original number of OPO in the co-cultured siphon tip explants and ORB. No changes were seen in the siphon tip explants, including the proximal margin lacking OPO, although they remained viable based on rhythmic muscular contractions. After 9–10 days in culture, some of the explants were fixed and flat mount preparations were stained with receptor cell markers (Figs. 7L–N). DIC microscopy showed that orange pigment cups were present (Fig. 7L), and rhodamine-phalloidin (Fig. 7M) and DAPI (Fig. 7N) staining showed that receptor cells differentiated in the regenerated OPO. Thus, complete OPOs can regenerate in mid-section siphon explants, indicating that the source of cells for OPO regeneration must be within the oral siphon. Lastly, the results demonstrate that OPO regeneration in cultured explants retains the polarity of *in vivo* regeneration.

Control of OPO number during regeneration

To investigate the fidelity of OPO regeneration, the oral siphon of animals with seven, eight, or nine OPOs was amputated, the regenerating animals were maintained individually, and the number of regenerated OPOs was determined at 8–10 dpa. After a single cycle of siphon regeneration, all of these animals regenerated the original number of OPOs (Table 1). Subsequently, OS amputation was repeated three additional times in the same animals to generate a total of four successive oral siphon amputation and regeneration cycles. The results showed that the OPOs regenerated with fidelity for at least three cycles of amputation (Table 1). The time required for OPO regeneration did not markedly increase or decrease with additional amputation cycles. The normal OPO pattern was disrupted after the fourth cycle: some animals regenerated fewer or more than the original OPO number (Table 1). It should be noted that the period between successive amputations in these experiments was insufficient to allow the oral siphon enough time to regenerate to full length. Thus, overall siphon length became shorter with every cycle, and by the fourth cycle the amputation plane was near or below the base of the oral siphon. The results suggest that OPO number tends to regenerate with fidelity but the precision of OPO patterning can be disrupted by amputation near the oral siphon base.

To directly investigate the relationship between the proximal–distal axis of the oral siphon and OPO regeneration, the number of regenerated OPO was investigated after amputation at the distal margin, middle, or base of the oral siphon (Fig. 8A). The basal amputations were done in the region below the major horizontal blood vessel and tentacle row, and sometimes included the distal margin of the branchial sac (Fig. 8A). The original number of OPOs was formed after amputation at the distal tip or middle of the oral siphon (Fig. 8D top and middle frames). After amputation at the siphon base, the number of OPO ranged from 1 to 16, but was frequently more than the typical eight (Fig. 8D, bottom frame). Fewer regenerated OPOs after basal amputation probably indicate retardation of regeneration, which was monitored at 8–10 dpa. However, the additional regenerated OPOs were caused by the formation of duplicate or triplicate orange pigment spots (Figs. 8B, C). Each duplicate or triplicate pigment spot originated within a single line of orange pigment cells above a single ORB. Some of the animals with duplicated orange pigment spots were followed to later stages of siphon regeneration. Each pigment spot produced a complete OPO with receptor cells surrounded by small siphon lobes, which increased from 8 to 16 in the most extreme cases (data not shown). These results suggest that the site of amputation along the proximal–distal axis of the oral siphon determines the pattern of OPO and siphon lobe regeneration.

To extend these results, two types of oblique amputations were done that intersected only a part of the structural divergent region at

Fig. 7. OPO regeneration in cultured oral siphon explants. (A) Left: A diagram illustrating the two-part amputation procedure used to produce oral siphon tip and mid-section explants. (A) Right: An animal immediately after the operations shown on the left with tip (top) and mid-section (middle) explants above the amputated oral siphon (bottom). The red line labeled 1 indicates the position of the first cut, and the red line labeled 2 indicates the position of the second cut. (B–I) Cultured oral siphon mid-section explants surrounded by tunic showing the reformation of orange pigment cells at the distal but not the proximal margin. Panels B, D, and H show explants viewed from their original distal ends. The boxes indicate 8×-magnified views showing part of the distal siphon margin in panels C, E, and I, respectively. Arrows indicate accumulations of orange pigment cells. (F) The same oral siphon explant as in panel D viewed from the original proximal margin, with a box indicating 8× magnification in panel G. The arrow in panel G shows the proximal margin of the siphon explant, which lacks accumulated orange pigment cells. The arrowhead in panel G shows a longitudinal column of orange pigment cells, which appears between the distal and proximal margins in some of the cultured explants. Panels B, D/F, and H are different OS explants. (J) A mid-section explant after 6 days in culture with tunic removed that has been cut along its proximal–distal axis and opened to show columns of intense orange pigment cells (arrowhead) located in the ORB. The arrow indicates the tentacles, which mark the proximal side of this explant. (K) A mid-section explant after 9 days in culture with tunic removed (bottom) that was co-cultured with a tip explant (top) from the same animal (see A). Arrowhead in panel K shows OPOs that appeared at the distal margin of the mid-section explant (bottom). No OPOs appeared at the proximal margins of the siphon tip (top) or mid-section explants (bottom) during the culture period. Scale bar in panels A and B is 200 μm; magnification is the same in panels B–K. Flat mount preparations of the cultured oral siphon explant shown in panel K (bottom) after 8 days in culture show complete regeneration of an OPO with an orange cup (L) containing rhodamine-phalloidin (M) and DAPI (N) stained receptor cells. Scale bar in panel L is 80 μm; magnification is the same in panels L–N.

the base of the oral siphon (Fig. 8E). In some of these oblique amputations, the amputation plane extended from the anterior tip to the posterior base of the oral siphon. In other oblique amputations, the amputation plane extended from the anterior base to the posterior tip of the oral siphon. Multiple orange pigment spots regenerated from both types of oblique amputations (Fig. 8H), but the distribution of duplicated OPO was not uniform (Figs. 8F, G). In both oblique amputations, multiple and more closely spaced orange pigment spots were observed on the side of the regenerated siphon margin originating from the base of the siphon, whereas single and more-widely spaced pigment spots developed along the margin originating from the distal and middle parts of the siphon (Figs. 8F, G). The results

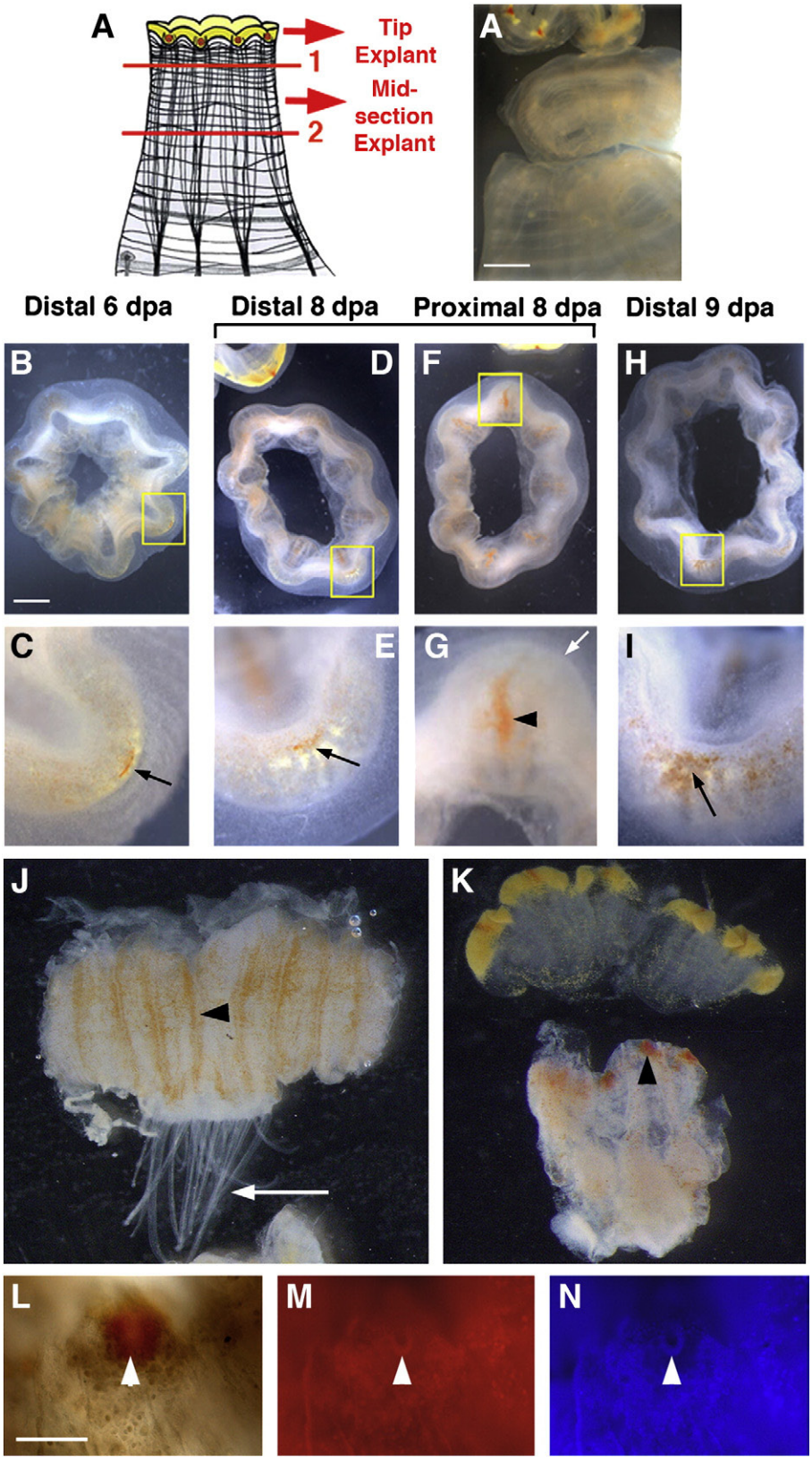

Table 1
Fidelity of OPO reformation during oral siphon regeneration.

Oral siphon amputation cycle[a]	Beginning OPO number	N[b]	Regenerated OPO number[c]				
			<7	7	8	9	>9
1	7	9	0	9	0	0	0
	8	13	0	0	13	0	0
	9	3	0	0	0	3	0
2	7	9	0	9	0	0	0
	8	13	0	0	11	0	0
	9	3	0	0	0	3	0
3	7	9	0	7	0	0	0
	8	11	0	0	11	0	0
	9	3	0	0	0	3	0
4	7	7	1	1	0	0	2
	8	11	0	0	2	1	7
	9	3	0	0	0	0	2

[a] Oral siphons were amputated successively as shown below and OPO regeneration was assayed at 8–10 dpa.
[b] *N* decreases between cycles due to mortality after oral siphon amputation.
[c] Regenerated OPO number does not include animals that died during the cycle.

show that OPO pattern is dependent on cues positioned along the proximal–distal axis of the oral siphon.

Roles of the nervous system and neural complex in OPO regeneration

The role of the nervous system and neural complex in OPO regeneration was determined in two types of experiments. First, siphon nerve replacement was examined by GFP fluorescence after oral siphon amputation in E15 transgenic animals (Fig. 9A). By 5-dpa new GFP-stained nerve processes were detected extending into the regenerating region from the region of pre-existing nerves in the remaining part of the siphon (Figs. 9B, C). Several days later, thin neurites appeared in the vicinity of the regenerating OPOs (Figs. 9D, E). These results suggest that nerve outgrowth occurs during in OPO regeneration. Second, siphon nerve and OPO regeneration was examined after the neural complex was removed (Figs. 9F, G). The neural complex consists of two parts: the cerebral ganglion, which contains most of the cell bodies of efferent nerves entering the siphons (Dahlberg et al., 2009), and the neural gland, which is proposed to be a homologue of the vertebrate periventricular organ (Deyts et al., 2006). In principle, one or both parts of the neural complex could be involved in siphon regeneration. The results showed that OPO numbers in regenerating animals lacking the neural complex did not differ from sham-operated and unoperated controls: eight OPO were usually regenerated in all cases (Fig. 9H), and the duration of the replacement process was not affected. We conclude that neither part of the neural complex is required for patterning of the OPO during oral siphon regeneration.

Discussion

The results of the present investigation resolve several major issues concerning OPO regeneration in the oral siphon of *C. intestinalis*. First, they define the sequence of oral siphon regeneration, showing that distal structures, such as the OPOs and siphon lobes, are reformed quickly, whereas overall oral siphon re-growth is completed more slowly by intercalary regeneration. Second, they indicate that OPOs are normally replaced by the local differentiation of progenitor cells within the oral siphon, respecting the original polarity of this structure, rather than by cell migration from distant sources within the body. Third, they reveal different potencies for regeneration along the proximal–distal axis of the oral siphon that control OPO and siphon lobe pattern at its distal extremity. Finally, the use of E15 enhancer trap line animals suggests the existence of a neural component within the OPO and the possibility of nervous system participation in oral siphon and OPO regeneration. These results provide new information concerning the mechanisms of regeneration in a solitary ascidian and suggest that *C. intestinalis* is an attractive model for chordate regeneration.

Oral siphon and OPO regeneration

Previous siphon regeneration studies in *Ciona* were focused primarily on general parameters of re-growth and development, such as the time required to complete regeneration (1–2 months) (Whittaker, 1975), the induction of extra siphons (Von Haffner, 1933), and the relative lengths and nutritional characteristics of the regenerated and original siphons (Fox, 1924; Wermel and Lopaschow, 1930). Although some of these studies recognized that OPO replacement was an early part of the OS regeneration program (Whittaker, 1975), the sequence in which OPO parts are reformed and the underlying processes of regeneration was not elucidated. For example, the relationship between normal oral siphon development and regeneration, whether the parts of the siphon reform with fidelity, and whether the potency for regeneration is the same throughout adult life are some of the issues that remained unknown. Furthermore, most previous studies focused on the oral siphon as a whole rather than on its parts.

We demonstrate here that oral siphon regeneration occurs in a sequence of well-defined steps. The first step involves closing and repair of the wounded siphon. The second step is hallmarked by formation of new orange pigment cups. Free orange pigment cells initially increase in number throughout the distal portion of the siphon but are most prominent in the ORB. Subsequently, many of the newly formed orange pigment cells become aligned at the margin of the regenerating siphon above distinct areas corresponding to the position of ORBs. The number of orange pigment lines corresponds to the number of ORBs and regenerated OPOs. Next, the orange pigment cells condense into spots, which are localized approximately in the center of each ORB. About this time, the precursors of receptor cells form crypts in the epidermis. The third step in oral siphon regeneration is replacement of the three parts of the OPO: the orange pigment cup, its putative neural component, and differentiated receptor cells. Based on phalloidin, DAPI, and GFP (in transgenic animals) expression, orange pigment cup formation is likely to precede the appearance of differentiated receptor cells. Accordingly, the following scenario is proposed for OPO development. First, epidermal receptor cell precursors buckle inward to form foci for condensation of underlying orange pigment cells into spots and cups. Second, the mesenchymal cells of the orange pigment cup induce terminal differentiation of receptor cells and their putative neural component, thus producing the complete OPO. The reformation of the siphon lobes, which occurs at the same time as OPO differentiation, may also play a role in this scenario. The fourth step in oral siphon regeneration is the formation of the marginal band of yellow pigment cells. Yellow pigment cells begin to accumulate as extensions or “wings” of the OPO, then gradually increase in number, extend laterally, and connect along the rim of the regenerating siphon. The source of the yellow pigment cells is uncertain, although their proximity to the OPO suggests that this structure may somehow be involved in their formation. The final step of regeneration is the continued outgrowth of the new siphon. We have shown that cell proliferation occurs in the distal region of the siphon, which accounts for siphon outgrowth during regeneration.

Our results demonstrate that the distal parts of the oral siphon, including the OPOs, siphon lobes, and yellow pigment band, are the first structures to be replaced after amputation and that their regeneration precedes replacement of the proximal zone. Thus, in this aspect, *Ciona* oral siphon regeneration resembles regeneration of the vertebrate limb, which initially reforms distal structures before

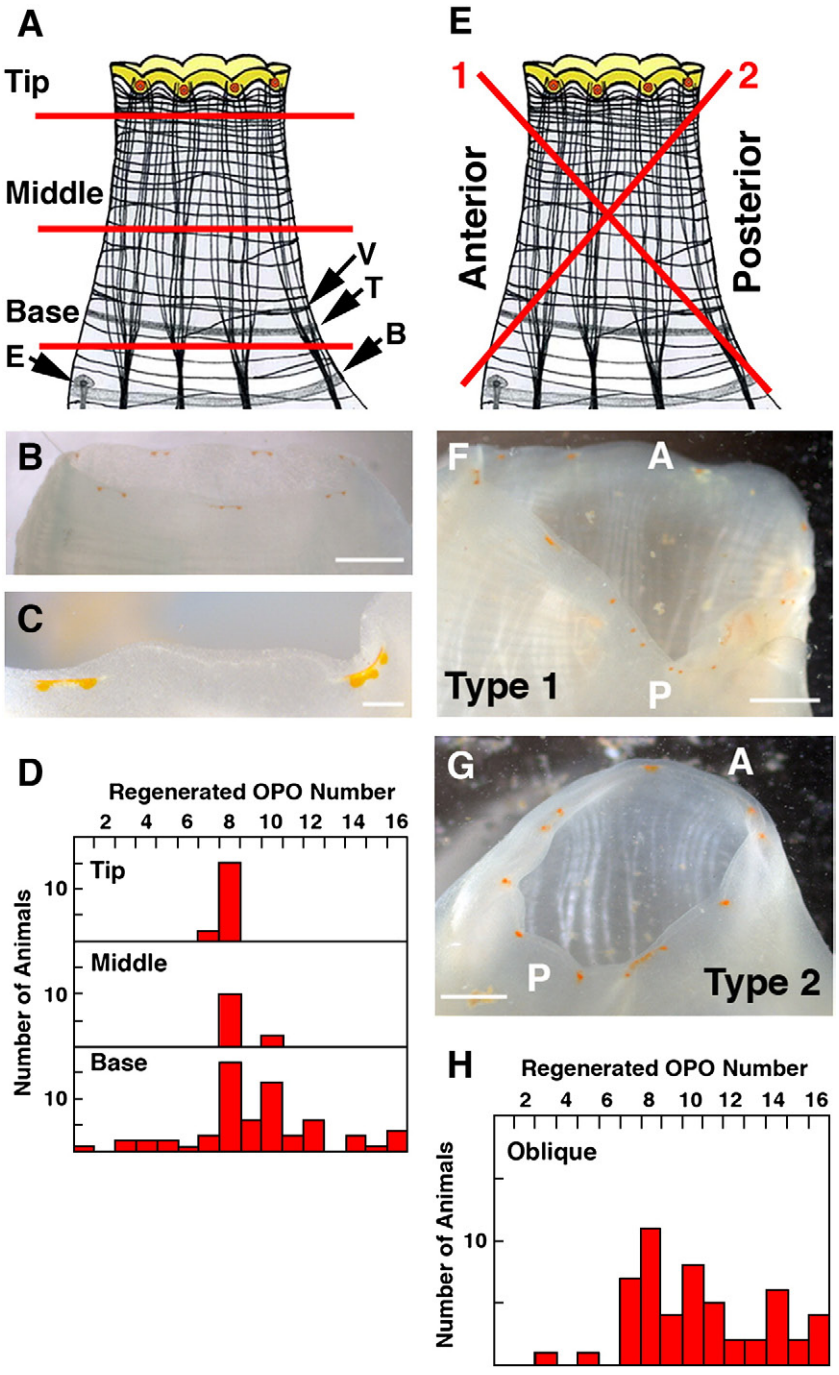

Fig. 8. The position of oral siphon amputation determines the pattern of OPO regeneration. (A) A diagram illustrating amputations in planes (red lines) perpendicular to the proximal–distal axis near the tip, middle, and base of the oral siphon. V, major vertical siphon blood vessel; T, row of siphon tentacles; B, distal edge of branchial sac; E, anterior hood of the endostyle. (B) Regeneration of multiple OPOs 8–10 days after amputation at the oral siphon base. (C) Magnified view (4×) of regenerated doublet and triplet OPOs. (D) Histograms showing the effect of amputation position (tip, middle, or base) on the number of regenerated OPOs. (E) A diagram illustrating oral siphon amputations in planes (red lines) oblique to the proximal–distal axis. Oblique plane 1 cut through the OS from the anterior tip to the posterior base. Oblique plane 2 cut through the OS from the posterior tip to the anterior base. (F, G) Multiple OPOs regenerate only on the side of the OS corresponding to the base (P in F or A in G) as a result of Type 1 (F) or 2 amputations (G). Scale bars in panels B, F, and G are 250 μm. (H) A histogram showing the number of regenerated OPOs in pooled animals after amputation in oblique planes.

more proximal components are replaced by intercalary regeneration (Nye et al., 2003; Echeverri and Tanaka, 2005).

OPO regeneration and development

The present study revealed a close relationship between OPO regeneration and development. It was previously shown that OPOs are formed from colorless precursors located below the edge of the oral siphon during juvenile development (Chiba et al., 2004). The pigment cell precursors may be descendants of the neural crest-like cells, which are known to produce pigment cells after migrating into the developing siphon from the dorsal midline of tadpole larvae (Jeffery, 2006; Jeffery et al., 2008). The results of Chiba et al. (2004) and our studies of later stages show that normal OPO development is strikingly similar to the sequence of events involved in OPO regeneration. During siphon development, the orange pigment spots move distally between each of the siphon lobes to form OPOs, and yellow pigment cells gradually extend lateral to the OPO to produce a

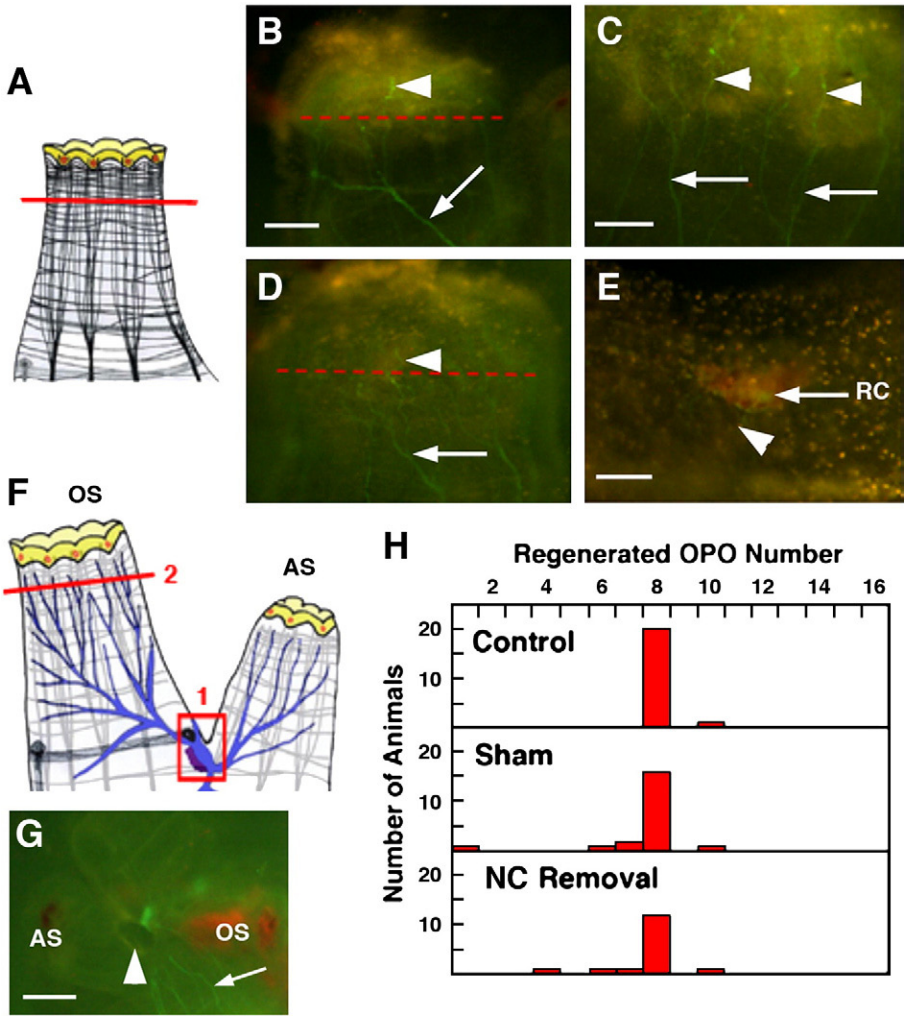

Fig. 9. The role of nervous system in OPO regeneration studied in E15 transgenic animals. (A) A diagram showing the position of oral siphon amputation used to examine nerve regrowth. (B–E) Nerve fiber reformation to the regenerating oral siphon. Nerves were detected in oral siphon regenerates by GFP fluorescence at 5 (B, C) and 7 (D, E) days after amputation. (B, D) Dashed red lines show the approximate boundary between the regenerating area and oral siphon stump. Arrowheads: nerve fibers in regenerating oral siphon. Unlabeled arrow: major oral siphon nerve. (C) Magnified region of oral siphon in panel B showing GFP-stained nerve fibers (arrowheads) in the regenerating area and pre-existing nerves (unlabeled arrows) proximal to them. (E) Magnified region of oral siphon from panel D showing GFP stained nerve fiber associated with receptor cells in a regenerating OPO (arrowhead). Scale bar in panel B is 100 μm; magnification is the same in panels B and D. RC, receptor cells. Scale bar in panel C is 50 μm. Scale bar in panel E is 20 μm. (F–J) Effects of neural complex removal on OPO reformation and oral siphon nerve regeneration. (F) A diagram illustrating the two-step procedure for neural complex removal and oral siphon amputation. The siphon nerves and neural complex (within the red box labeled 1) depicted in blue. In the first step (red box labeled 1) the neural complex was removed, and in the second step (red line labeled 2) the oral siphon tip was removed from the same animal. (G) An operated E15 transgenic animal in which the neural complex was removed (arrowhead) showing severed GFP-positive nerve tracts (arrow). OS, oral siphon; AS, atrial siphon. Scale bar in panel G is 500 μm. (H) Regeneration of the normal OPO number after distal oral siphon amputation in controls containing a neural complex (top), sham-operated animals in which the neural complex was dissected free of surrounding tissue but not removed (middle), and animals lacking a neural complex (NC).

yellow pigment band along the circumference of the oral siphon. Thus, at least morphologically, OPO regeneration appears to recapitulate OPO development.

Oral siphon and OPO regeneration decrease with age

Using a large size range of regenerating animals (2–15 cm), we have demonstrated a negative correlation between animal length and the rate of OPO regeneration. Animal length is a rough estimate of age in *C. intestinalis*, which have a total life span in the wild of about a year (Berrill, 1947; Millar, 1952; Dybern, 1965). We found that small young animals can regenerate OPOs as soon as 2–3 dpa, three-four times more rapidly than the largest old animals. Since the OPO precursors are produced locally in the oral siphon (see below), the decreased regeneration rate in larger animals could not reflect the longer distance needed by progenitor cells to migrate into the siphon from a regeneration center in the body. Rather, this observation suggests a reduction in the capacity to replace lost parts during aging, as typically seen in regenerating frog tadpole limbs (Muneoka et al., 1986; Givan et al., 2002). In contrast to frogs and most other vertebrates, however, even the largest (and presumably the oldest) *Ciona* we have been able to obtain can fully regenerate their OPO given enough time, suggesting that there may be no point during the life cycle in which regeneration completely ceases. A negative correlation similar to that we have described for OPO regeneration has also been observed between age (size) and neural complex regeneration (Dahlberg et al., 2009), suggesting an overall reduction in the capacity to regenerate missing body parts with age in *Ciona.*

OPO regeneration is controlled by local cell differentiation

Hirschler (1914) concluded that organs in the ventral portion of the body, including the gonads, heart, stomach, and at least a fragment of the pharynx (branchial sac), are required for siphon regeneration in *Ciona*. Accordingly, a role for long distance migration of stem/precursor cells, perhaps via the extensive sinuses of the circulatory

system (Millar, 1953), might have been expected for OPO regeneration. On the contrary, we have demonstrated that local cell differentiation, rather than long distance migration, is a key factor in OPO replacement. Several lines of evidence support this conclusion. First, when regions containing proliferating cells at the distal margin of the regenerating oral siphon were UV irradiated, OPO and siphon regeneration was blocked. Second, OPO regeneration occurred normally after a region in the middle of the siphon was UV irradiated. This manipulation would have been expected to impede cell migration from proximal sources. Third, the strongest evidence against a role for long distance migration was provided by siphon explant experiments. These studies demonstrate that complete OPO can regenerate from isolated parts of the oral siphon without the participation of any other part of the body. Furthermore, the proximal–distal polarity of OPO regeneration was respected *in vitro*: OPO formed only on the original distal surface of the explants. Polarity of OPO regeneration is a common property uniting *Ciona* with many other regenerating systems (Reddien and Sánchez Alvarado, 2004; Brockes and Kumar, 2008). We also found that each OPO can be regenerated as a unit independent of other OPOs. This idea was supported by the results of unilateral UV irradiation and most conclusively by microcautery, which showed that a single OPO could regenerate after it was removed from the edge of the oral siphon.

Based on these results, we propose a different scenario for oral siphon and OPO regeneration than originally suggested by Hirschler (1914). Rather than long distance migration from a source outside the siphon, we suggest that stem/progenitor cells responsible for reforming the OPOs are located in niches within the siphon. Nevertheless, in some instances, cells outside of the siphon can clearly be recruited to reproduce OPO (albeit forming abnormal patterns; see below), such as when the oral siphon is amputated at its base. It will be interesting to determine the source of OPO progenitors in this special case of oral siphon regeneration.

Regulation of OPO number during regeneration

The *Ciona* oral siphon usually has eight OPOs, although a few animals have seven or nine OPOs. We have shown that OPO number is reproduced with fidelity through three successive cycles of amputation along the length of the siphon up to its base, suggesting that the mechanism for generating the normal pattern of OPOs is robust and probably genetically determined. Additionally, no speeding of the OPO regeneration process was seen after three successive siphon amputations, indicating that a previous injury does not "prime" the regeneration mechanism. In contrast, we have found that OPO number increases when the oral siphon is amputated near or below its base. Hirschler (1914) also found more or less than the normal number of pigment organs in regenerated oral and atrial siphons after amputation of the entire dorsal part of the body, including the neural complex. Because large variations in the diameters of regenerated siphons were noted under these conditions, he concluded that the number of OPOs is related to the circumference of the siphon aperture. We have come to a different conclusion. Namely, the number of regenerated OPOs is dependent on the width of ORBs (oral siphon regeneration bands) along the proximal–distal axis of the siphon.

Our model for the role of ORB in OPO regeneration is illustrated in Fig. 10. It is proposed that stem/progenitor cell niches responsible for OPO regeneration are located in ORB running along the proximal–distal axis of the oral siphon. The ORBs are limited in width by adjacent LMBs. When the ORB is narrow, such as in the distal and middle portions of the oral siphon, a single OPO is regenerated from each ORB, but as the LMBs taper and the ORBs correspondingly widen near the siphon base, multiple OPOs can be regenerated within a single ORB. The manner in which duplicate and triplicate OPOs are formed is interesting. They are split off from single long lines of orange pigment cells, each forming duplicate or triplicate OPOs above a single ORB. The spatial cues that induce orange pigment cells to condense into single or multiple spots near the margin of the siphon are unknown and worthy of further investigation.

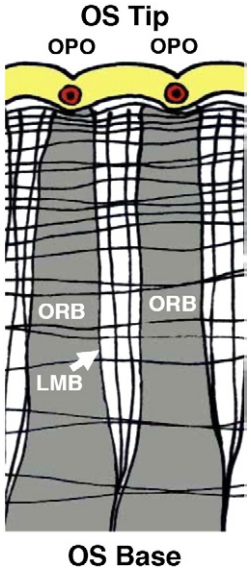

Fig. 10. A diagram illustrating the relationship between OPO location and oral siphon regeneration bands hypothesized to control the number of regenerated OPO after siphon amputation at different positions along the proximal–distal axis. OPO, oral pigment organ; LMB, longitudinal muscle band; OS, oral siphon; ORB, OPO regeneration band.

The ORB model is supported by several different experimental results. First, the number of regenerated OPOs is replicated with fidelity when amputations intersect the relatively narrow ORBs in the distal and middle portions of the oral siphon. However, amputations near the siphon base, where the ORBs are wider, frequently result in regeneration of multiple OPOs. It should be emphasized that the latter situation would also have occurred when the entire dorsal portion of an animal was severed below the bases of both siphons (Hirschler, 1914). Second, siphon amputations in oblique planes also generated multiple OPOs when they intersected part of the structurally divergent region near the siphon base. Third, in mid-section siphon explants, a large number of orange pigment cells differentiated in vertical columns within the ORBs, presumably because the original OPOs at the distal margin normally exert a negative influence on further pigment cell differentiation, which no longer operates in mid-section explants. These results suggest that the pre-existing structure along the proximal distal axis of the siphon is important in controlling OPO number during oral siphon regeneration.

Are OPO photoreceptors?

Previous studies have provided evidence both for and against the possibility that OPOs are photoreceptors (Hecht, 1918; Millar, 1953; Dilly and Wolken, 1973). The primary evidence supporting photoreceptor function is OPO structure, which resembles a ciliated light sensory organ (Dilly and Wolken, 1973). Evidence against this function is that siphon amputation does not affect the orientation of the animal toward light (Hecht, 1918), although this argument has been countered by pointing out that there may be redundant photosensitive organs in other parts of the body. A critical point in deciding whether OPOs are sensory organs of any type is their connectivity to the central nervous system. Although some previous studies claim that OPOs are connected to the neural ganglion by siphon nerves (Markman, 1958; also see Dilly and Wolken, 1973), inspection of these data indicates that direct evidence is lacking. Unfortunately, *Ciona* siphon nerves are very fine and lack myelination,

making them difficult to trace by conventional staining methods. In ascidian species in which siphons nerves can be seen more readily, connections to OPOs are not apparent (Mackie et al., 2006).

Our results with animals of the E15 transgenic line, which expresses GFP throughout the nervous system (Awazu et al., 2007; Dahlberg et al., 2009), have clarified this picture. First, we have shown that the inner portion of the orange pigment cup containing ciliated receptor cells fluoresces brightly in E15 transgenic animals, suggesting that they may be associated with a basal neural structure. Second, GFP-fluorescing nerve fibers were seen in the region just below regenerating OPOs, to which they appeared to enter. The cell bodies of these neurites may either be outgrowths of the severed siphon nerves, which re-extend into the regenerating siphon, or neurite extensions of the receptor cells. Although the photoreceptor function of OPOs needs further investigation, their neural connectivity, putative neural components, and rapid regeneration suggest an important neurophysiological role.

Role of the nervous system in OPO organization and regeneration

Nerves are required for newt limb regeneration (Brockes, 1987; Kumar et al., 2007), and the reformation of the siphon nerves could also be a critical factor in replacing *Ciona* OPOs and siphons. Our results showed that the number of regenerated OPOs was not affected by excision of the neural complex prior to siphon amputation and OPOs were replaced as rapidly after amputation in animals lacking the neural complex as in sham-operated controls. Thus, the neural complex does not appear to be involved in OPO regeneration. Although the neural complex itself is capable of regeneration in *Ciona* (Dahlberg et al., 2009), this process is not very advanced by the time complete OPOs are detected and thus seems to be irrelevant to our conclusions. These results are also confirmed by complete OPO regeneration in oral siphon explants, which lack the neural complex.

The ability of new OPOs to regenerate with fidelity after neural complex removal could be related to the persistence and regeneration of siphon nerves in the portion of the oral siphon remaining after amputation, as we have demonstrated in E15 transgenic animals. Whereas the cell bodies of most siphon nerves are located within the cerebral ganglion, Dahlberg et al. (2009) showed that a few cell bodies may be located outside this structure. Perhaps these neurons could be extended to reform siphon nerves during the regeneration of animals lacking the cerebral ganglion. Further studies will be required to determine whether the neural complex is required for regeneration of these siphon nerves. Transgenic *Ciona* will be excellent subjects to study the role of the nervous system in oral siphon and OPO regeneration.

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